FINAL PROGRESS REPORT	GRANT NUMBER 2 R01 GM061964-04	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR	PERIOD COVERED BY THIS REPORT	
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APPLICANT ORGANIZATION	9/01/1999	8/31/2006
Georgia Tech Research Corporation		

TITLE OF PROJECT *(Repeat title shown in item 1 on first page)*

Caspase Inhibitors

Specific Aims

1. Design and synthesize α-ketoamide transition-state inhibitors with selectivity for caspase-3, caspase-6, and caspase-8.
2. Design and synthesize potent and specific aza-epoxide inhibitors for caspase-3, caspase-6, and caspase-8.
3. Synthesize libraries of aza-epoxide caspase inhibitors.
4. Assay all new inhibitors for specificity with available caspases and other enzymes.
5. Assay a few potent inhibitors for their ability to inhibit apoptosis.

Progress Report

Period Covered: September 1, 1999 to August 31, 2006.

Summary. Caspases are members of the CD clan of cysteine proteases. Other members of the clan include clostripain, legumain, gingipain, and separase. During this research, *we have discovered three new classes of irreversible inhibitors: aza-peptide epoxides, aza-peptide Michael acceptors, and allyl sulfones*. The aza-peptide epoxides and aza-peptide Michael acceptors are highly specific for clan CD cysteine proteases. They have little or no inhibitory potency toward clan CA cysteine proteases (papain, calpain, and cathepsin B), serine proteases, aspartate proteases, or the proteasome. Within clan CD cysteine proteases, aza-peptide epoxides and aza-peptide Michael acceptors show high specificity toward their targeted clan CD protease. Thus, caspase inhibitors don't react with legumain or gingipain, while legumain inhibitors don't react with caspases or gingipain. Considerable selectivity for individual caspases has been obtained by changing both the P and P' residues. Allyl sulfones, which we have not developed extensively, appear to be selective for the clan CA cysteine proteases. Aza-peptide epoxides and aza-peptide Michael acceptors appear to be *the most selective classes of inhibitor for caspases and other clan CD cysteine proteases*. Previously described inhibitors for caspases have been shown to inhibit effectively clan CA cysteine proteases such as the cathepsins (Rozman-Pungercar, Kopitar-Jerala et al. 2003). Thus, our new inhibitors have great potential for elucidating the biological function of clan CD cysteine proteases and for therapeutic uses. In addition to clan CD cysteine protease, we discovered late in the project that aza-peptide epoxides were inibitors for the main protease from the SARS coronavirus. Unfortunately, we were unable to pursue this new avenue of research since this grant was not renewed.

Patents and Patent Applications:

Propenoyl Hydrazides, J. C. Powers, O. D. Ekici, M. G. Götz, K. E. James, Z. Z. Li, and B. Rukamp, provisional patent application filed February 18, 2004, patent application filed February 18, 2005.

Aza-Peptide Epoxides, J. C. Powers, J. Gheura, K. A. Ellis, Z. Z. Li, (2006) U. S. Patent 7,056,947, June 6, 2006. Invention occurred prior to grant, but was developed during the period of grant.

Aza-Peptide Epoxides CIP, J. C. Powers, J. Glass, filed January 24, 2006.

Peptidyl Allyl Sulfones, J. C. Powers and M. Götz, provisional patent application filed November 9, 2004, patent application filled in November 2005.

Publications:

Design, Synthesis, and Evaluation of Aza-Peptide Michael Acceptors as Selective and Potent Inhibitors of Caspases-2, -3, -6, -7, -8, -9, and -10, Ekici, O. D., Li, Z. Z., Campbell, A. J., James, K. E., Asgian, J. L.,

Mikolajczyk, J., Salvesen, G. S., Ganesan, R., Jelakovic, S., Grütter, M. G. and Powers, J. C. (2006) *Biochemistry* 49, 5728-5749.

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Aza-Peptide Michael Acceptors: A New Class of Inhibitors Specific for Caspases and Other Clan CD Cysteine Proteases, Ekici, O, D., Götz, M. G., James, K. E., Li, Z. Z., Rukamp, B. J., Asgian, J. L., Caffrey, C. R., Hansell, E., Dvorák, J., McKerrow, J. H. Potempa, J., Travis, J., Mikolajczyk, J., Salvesen, G. S., Powers, J. C. (2004) *J. Med. Chem.*, 47, 1889-1892.

Design, Synthesis, and Evaluation of Aza-Peptide Epoxides as Selective and Potent Inhibitors of Caspases-1, -3, -6, and -8, James K. E., Asgian, J. L., Li, Z. Z., Ekici, O. D., Rubin, J. R., Mikolajczyk, J., Salvesen, G. S., and Powers, J. C. (2004) *J. Med. Chem., 47*, 1553-1574.

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Dipeptidyl Peptidase I: Importance of Progranzyme Activation Sequences, Other Dipeptide Sequences, and the N-Terminal Amino Group of Synthetic Substrates for Enzyme Activity, Tran, T. V. Ellis, K. A., Kam, C. M., Hudig, D., and Powers, J. C. (2002) *Arch. Biochem. Biophys. 403*, 160-170.

Inhibition of Human Neutrophil Elastase by Erythromycin and Flurythromycin, Two Macrolide Antibiotics, Gorrini, M., Lupi, A., Viglio, S., Pamparana, F., Cetta, G., Ladarola, P., Powers, J. C., and Luisetti, M. (2001) *Am. J. Resp. Cell Mol. Biol. 25*, 492-499.

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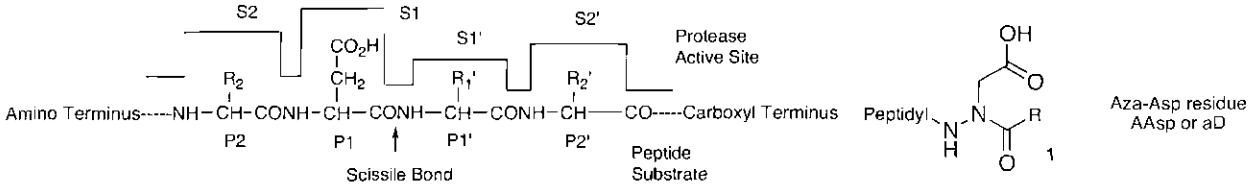
Nomenclature. We will use the subsite nomenclature of Schechter & Berger (Schechter and Berger 1967) to describe the individual residues of the inhibitors and the corresponding subsites of the enzyme. The primary substrate binding site of caspases is S1, which recognizes the P1 Asp residue in substrates and inhibitors. The nomenclature is shown in the following figure. In this proposal we refer to aza-peptides as inhibitor structures in which the P1 amino acid residue is replaced by an aza-amino acid where the α-carbon of the amino acid is replaced by a nitrogen atom. An aza-aspartic acid residue is shown below (**1**). In peptide sequences this will be abbreviated AAsp or aD when using the amino acid one letter code. Other abbreviations used in this proposal are listed at the end of the Research Plan (Page 57).



Specific Aims. The specific aims of our prior proposal are listed above. We made good progress on all of these specific aims. In the course of this research, we discovered that aza-peptide inhibitors are highly specific for clan CD cysteine proteases. Although the original specific aims only proposed work with caspases, we have extended the aza-peptide design and the scope of our research to other clan CD cysteine proteases, particularly legumains and gingipains, as these enzymes have been described in the literature and have become available for kinetic inhibition assays during the period of this grant. We also extended the design to the main protease from the SARS coronavirus.

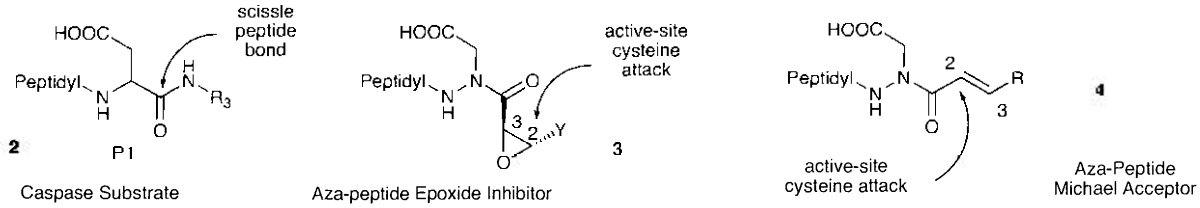
α-Ketoamide Calpain Inhibitors (Specific Aim 1). The PI's laboratory has previously synthesized a variety of dipeptidyl and tripeptidyl α-keto esters, α-keto amides, and α-keto acid transition-state inhibitors of the cysteine proteases calpain I and II (Li, Patil et al. 1993; Li, Ortega-Vilain et al. 1996). These compounds are potent inhibitors of both calpain I and calpain II with K_i values as low as 0.020 μM. One of these compounds (AK295) has been studied extensively in stroke and head injury models and is effective at preventing peripheral neuropathy (collaboration with J. Glass, Emory University (Wang, Davis et al. 2004)). With support from BASF in the mid-1990s, the PI's research group synthesized over 50 peptide α-ketoamides with P1 Asp residues as inhibitors for caspase 1. Many of the compounds are potent caspase 1 inhibitors with K_i values in the low nanomolar range (i.e., Ac-Tyr-Glu-Val-Asp-CONHCH$_2$CH$_2$Ph, K_i = 0.4 nM). During this grant period, we extended this inhibitor design to caspase 3, 6, and 8. We synthesized a number of new tetrapeptide ketoamides which are listed in Table 4. The compounds are slow-binding transition-state inhibitors of caspases 3, 6, and 8 and both initial and final K_i values in μM are reported also in Table 4.

Table 4.

	Caspase 3		Caspase 6		Caspase 8	
	$K_{i(initial)}$	$K_{i(final)}$	$K_{i(initial)}$	$K_{i(final)}$	$K_{i(initial)}$	$K_{i(final)}$
Z-Val-Glu-Val-Asp-CO-NH-(CH$_2$)$_2$Ph	0.317 μM	0.243	0.126	0.080	0.042	0.029
Z-Leu-Glu-Thr-Asp-CO-NH-CH$_2$Ph	3.638	0.762	0.968	0.156	0.125	0.011
Z-Leu-Glu-Thr-Asp-CO-NH-(CH$_2$)$_2$Ph	2.588	0.564	1.191	0.153	0.187	**0.003**
Z-Leu-Glu-Thr-Asp-CO-NH-(CH$_2$)$_3$Ph	3.249	0.733	1.972	0.461	0.120	0.013
Z-Leu-Glu-Thr-Asp-CO-NH-(CH$_2$)$_3$OCH$_3$	2.905	0.679	1.619	0.363	0.094	0.006

Four inhibitors utilized the predicted optimal sequence for caspase 8, Cbz-Leu-Glu-Thr-Asp. The other had a caspase 6 optimal sequence, Cbz-Val-Glu-Val-Asp. All four of the Cbz-Leu-Glu-Thr-Asp α-keto amides were potent inhibitors of caspase 8, with $K_{i(final)}$ values ranging from 3 nM to 13 nM. Interestingly, the compound design based on the caspase 6 optimal sequence, Z-Val-Glu-Val-Asp-CO-NH-(CH$_2$)$_2$Ph, was also an extremely potent inhibitor of caspase 8 with a $K_{i(final)}$ value of 29 nM. The most potent compound of the series was Cbz-Leu-Glu-Thr-Asp-CO-NH-(CH$_2$)$_2$Ph, which had a $K_{i(final)}$ of 3 nM, and was twice as potent as the next best inhibitor, Z-Leu-Glu-Thr-Asp-CO-NH-(CH$_2$)$_3$OCH$_3$ ($K_{i(final)}$ = 6 nM). Several of the inhibitors demonstrated selectivity between the three caspases studied. Cbz-Leu-Glu-Thr-Asp-CO-NH-(CH$_2$)$_2$Ph was 51 times more potent against caspase 8 than caspase 6, and 188 times more potent than against caspase 3. The methoxypropyl tetrapeptide derivative showed similar selectivity, with a 61-fold greater potency against caspase 8 than caspase 6, and a 113-fold greater potency than shown against caspase 3. We found that α-ketoamides with caspase specific sequences were difficult to synthesize and, thus, devoted most of our efforts toward aza-peptide inhibitors.

Aza-peptide Inhibitors (Specific Aim 2, 3, and 4). Aza-peptide epoxides were designed based on the structure of a good peptide substrate (**2**) with the placement of the carbonyl group of the epoxide moiety in a location identical to that of the carbonyl of the scissile bond in a substrate (**3**). Replacement of the α-carbon of the amino acid residue at P1 with a nitrogen results in the formation of an aza-amino acid residue. For example, in the design of the aza-peptide epoxide inhibitors for caspases, the α-carbon of the P1 Asp residue was converted to a nitrogen atom to obtain an aza-Asp residue (which will be abbreviated as AAsp). This novel group of irreversible inhibitors was found to be highly specific for cysteine proteases of clan CD, particularly caspases (Asgian, James et al. 2002; James, Asgian et al. 2004). Once we discovered the high selectivity of these inhibitors, we extended the design and synthesized aza-peptide Michael acceptor inhibitors (**4**) which are also selective for clan CD cysteine proteases (Ekici, Gotz et al. 2004).



We have synthesized, thus far, over 40 aza-peptide epoxide caspase inhibitors. Table 5 shows representative kinetic data obtained with caspase 1, 3, 6 and 8. The reported second order inhibition rates (k_2 values in $M^{-1}s^{-1}$) with caspases 1, 3, 6, and 8 ranged up to 1,910,000 $M^{-1}s^{-1}$ (James, Asgian et al. 2004). In general, the caspase 1 sequence Val-Ala-AAsp was most reactive with caspase 1, the caspase 3 sequence Asp-Glu-Val-AAsp was most reactive with caspase 3, the sequence Ile-Glu-Thr-AAsp was most reactive with caspase 6, and the caspase 8 sequence Leu-Glu-Thr-AAsp was most reactive with caspase 8. In table 5, we abbreviate the aza-aspartic acid as AAsp and the epoxide as EP. To understand the specificity and selectivity of the inhibitors at the P' position, we have changed the substituents on the epoxide moiety (**3**) from a simple alkyl groups (Y = CH_2CH_2Ph) to esters (Y = $COOR_4$), amides (Y = $CONHR_5$) or amino acids (Y = CO-AA-R_6). We sought to obtain increased selectivity by utilizing interactions with the S' subsites of the various caspases. Some of the more specific inhibitors for individual caspases are shown in bold in Table 5. The compound numbers are from our publication (James, Asgian et al. 2004).

Table 5.			caspase 1	caspase 3	caspase 6	caspase 8
23c	PhPr-Val-Ala-AAsp-EP-COOEt	*S,S*	32,200 $M^{-1}s^{-1}$	1040	75	1570
		R,R	6290	NI	NI	NI
23g	PhPr-Val-Ala-AAsp-EP-CONHCH$_2$Ph	*S,S*	**65,900**	1610	155	9360
		R,R	1630	NI	NI	NI
23l	PhPr-Val-Ala-AAsp-EP-CO-Phe-NH$_2$	*S,S*	32,700	625	65	390
		R,R	330	NI	NI	NI
25c	Cbz-Asp-Glu-Val-AAsp-EP-COOEt	*S,S*	11,800	1,070,000	5440	95,500
		R,R	4320	464,000	475	785
25d	Cbz-Asp-Glu-Val-AAsp-EP-COOCH$_2$Ph	*S,S*	54,700	**1,910,000**	12,700	188,000
26c	Cbz-Leu-Glu-Thr-AAsp-EP-COOEt	*S,S*	25,700	3210	8620	**61,200**
		R,R	19,600	3250	4390	34,700
26d	Cbz-Leu-Glu-Thr-AAsp-EP-COOCH$_2$Ph	*S,S*	43,500	3520	10,300	72,700
27d	Cbz-Ile-Glu-Thr-AAsp-EP-COOCH$_2$Ph	*S,S*	45,800	9500	86,200	58,500
		R,R	13,000	3110	**45,400**	4560

PhPr = $PhCH_2CH_2CO$-, NI = no inhibition, EP = epoxide (-C_2H_2O-), AAsp = aza-Asp, Cbz = $PhCH_2CO$-.

Stereochemistry. The epoxide moiety in aza-peptide epoxides has two chiral centers and the stereochemistry at these centers plays an important role in the potency of the inhibitor. In general, the order of reactivity of aza-peptide epoxides was *S,S* > *R,R* > *trans* > *cis* (Table 5). In comparing the rates of inhibition of caspase 1 by the *S,S*/*R,R* pairs (PhPr-Val-Ala-AAsp-EP-Y), we observed that the S,S isomer was more reactive by a factor of 5 to 100. With caspase 3, a comparison of the *S,S*/*R,R* pair for Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**) gave a ratio of 2. With caspase 6, comparison of the *S,S*/*R,R* pairs (Cbz-Ile-Glu-Thr-AAsp-EP-Y) gave ratios of 1 to 2. With caspase 8, comparison of the *S,S*/*R,R* pairs (Cbz-Leu-Glu-Thr-AAsp-EP-Y and Cbz-Ile-Glu-Thr-AAsp-EP-Y) gave ratios of 2 to 13. Thus, it appears that the epoxide stereochemistry was less significant with caspases 3, -6, and -8 than with caspase 1. Interestingly, some of the *R,R* epoxides while being less potent are actually more selective than the *S,S* epoxides. Almost all of the caspase 1 aza-peptide epoxides

with *R,R* stereochemistry (PhPr-Val-Ala-AAsp-(2*R*,3*R*)-EP-Y, **23**) reacted only with caspase 1, although at slow rates, and showed no inhibition of caspases 3, -6, and -8 (Table 5). The *R,R* isomer of **25c** (Cbz-Asp-Glu-Val-AAsp-EP-COOEt) was almost as reactive an inhibitor with caspase 3 as the *S,S* isomer, but was much more selective and was 105-fold, 975-fold, and 590-fold more reactive with caspase 3 than with caspase 1, -6, and -8, respectively.

Caspase and Clan CD Selectivity (Specific Aim 4). As expected, overall the most potent inhibitors had the preferred substrate sequence for the target caspase. The most potent caspase 1 inhibitors, such as PhPr-Val-Ala-AAsp-(*S,S*)-EP-CONHCH$_2$Ph (**23g**), have the optimal caspase 1 peptide recognition sequence PhPr-VAD. The most potent caspase 3 inhibitors, such as Cbz-Asp-Glu-Val-AAsp-(*S,S*)-EP-COOCH$_2$Ph (**25d**), have the optimal caspase 3 peptide recognition sequence DEVD. In addition, the most potent caspase 6 inhibitors, such as Cbz-Ile-Glu-Thr-AAsp-(*S,S*)-EP-COOCH$_2$Ph (**27d**), have the optimal caspase 6 peptide recognition sequence IETD. The only exception was caspase 8, where the caspase 3 tripeptide (Cbz-Glu-Val-AAsp-(*S,S*)-EP-COOEt) with the EVD sequence was better than the caspase 8 peptide recognition sequence LETD (**26d**, Cbz-Leu-Glu-Thr-AAsp-(*S,S*)-EP-COOCH$_2$Ph).

In general, the inhibitors designed for each caspase do inhibit other caspases somewhat, just not as potently as the one for which they were designed. The most specific, although not the most potent, inhibitor for caspase 1 was PhPr-Val-Ala-AAsp-(*S,S*)-EP-CO-Phe-NH$_2$ (**23l**, k_2 = 32,700 $M^{-1}s^{-1}$) which was 52-fold, 504-fold, and 84-fold more reactive with caspase 1 than caspase 3, caspase 6, and caspase 8, respectively. The most specific and still quite reactive inhibitor for caspase 3 was Cbz-Asp-Glu-Val-AAsp-(*R,R*)-EP-COOEt (**25c**, k_2 = 464,000 $M^{-1}s^{-1}$) which was 107-fold, 977-fold, and 591-fold more reactive with caspase 3 than caspase 1, caspase 6, and caspase 8, respectively. The most specific inhibitor for caspase 6 was Cbz-Ile-Glu-Thr-AAsp-(*R,R*)-EP-COOCH$_2$Ph (**27d**, k_2 = 45,400 $M^{-1}s^{-1}$, a moderate inhibitor) which was 3.4-fold, 14.5-fold, and 10-fold more reactive with caspase 6 than caspase 1, caspase 3, and caspase 8, respectively. The most specific for caspase 8 was Cbz-Leu-Glu-Thr-AAsp-(*S,S*)-EP-COOEt (**26c**, k_2 = 61,200 $M^{-1}s^{-1}$, a moderate inhibitor) which was 2.3-fold, 19-fold, and 7-fold more reactive with caspase 8 than caspase 1, caspase 3, and caspase 6, respectively.

To show that aza-peptide epoxides designed for caspases do not react with other clan CD enzymes, some of our caspase inhibitors were also tested with the clan CD enzyme legumain by W. Carter and Dr. Barrett at the Strangeways Research Institute, UK. The aza-peptide epoxides designed for caspases were specific for caspases as the $k_{obs}/[I]$ values with legumain ranged from 15 - 86 $M^{-1}s^{-1}$ for inhibitors **23d** (PhPr-Val-Ala-AAsp-*trans*-EP-COOCH$_2$Ph), **24c** (Cbz-Glu-Val-AAsp-(*S,S*)-EP-COOEt), **25c** (Cbz-Asp-Glu-Val-AAsp-(*S,S*)-EP-COOEt), and **26c** (Cbz-Leu-Glu-Thr-AAsp-EP-COOEt, both isomers) (Asgian, James et al. 2002). More importantly, two inhibitors, **23a** (PhPr-Val-Ala-AAsp-*trans*-EP-CH$_2$CH$_2$Ph) and **25c** (Cbz-Asp-Glu-Val-AAsp-(*R,R*)-EP-COOEt), showed no reactivity with legumain at all, as more than 90 % of the legumain activity remained after a ten minute incubation period (inhibitor concentrations ranged from 100 μM to 100 nM). In contrast, aza-peptide epoxides, such as Cbz-Ala-Ala-AAsn-(*S,S*)-EP-COOEt, which were designed with sequences specific for legumain, were potent legumain inhibitors with second-order rate constants ($k_{obs}/[I]$ values) up to 43,000 $M^{-1}s^{-1}$.

Specificity With Non-Clan CD Proteases (Specific Aim 4). The aza-peptide epoxides designed for caspases show little to no inhibition of the clan CA cysteine proteases papain, cathepsin B, and calpain. The inhibitors PhPr-Val-Ala-AAsp-EP-CH$_2$CH$_2$Ph (**23a**), Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**, both isomers), and Cbz-Leu-Glu-Thr-AAsp-EP-COOEt (**26c**, both isomers) at 208 μM showed no inhibition of papain and cathepsin B after 80 minutes of incubation. The inhibitor PhPr-Val-Ala-AAsp-*trans*-EP-COOCH$_2$Ph (**23d**, 208 μM) had a second order rate constant ($k_{obs}/[I]$) of < 10 $M^{-1}s^{-1}$ with papain and cathepsin B after 80 minutes of incubation. Our caspase inhibitors do not inhibit clan CA cysteine proteases, but we also wanted to determine if aza-peptide epoxides designed with sequences specific for clan CA enzymes were reactive. Examples of these compounds are Ac-Leu-ALeu-EP-COOEt, Cbz-Phe-ALeu-EP-COOEt, Cbz-Leu-AAbu-EP-COOEt, Cbz-Leu-AHph-EP-COOEt, and Cbz-Leu-Leu-ALeu-EP-COOEt. Inhibition constants ($k_{obs}/[I]$) were less than or equal to 20 $M^{-1}s^{-1}$ (unpublished results) with papain, cathepsin B, and calpain. The aza-peptide epoxide Suc-Np2-ALeu-*trans*-EP-COOEt (where Np2 = 2-naphthylalanine) was designed for cathepsin F and tested by Dr. Brömme at the Mount Sinai School of Medicine with cathepsins F, K, L, and S. Time-dependent inhibition was not observed and the inhibition ranges were greater than 100 μM for cathepsins K and L, and between 50-100 μM for cathepsins F and S. Clearly, this aza-peptide epoxide design is specific for clan CD cysteine proteases.

The aza-peptide epoxide Cbz-Leu-Leu-ALeu-EP-COOEt was actually designed to inhibit the proteasome, a threonine protease. This compound, tested by Dr. Orlowski at Mount Sinai School of Medicine,

showed no inhibition of the proteasome. It was also important to test for specificity against serine proteases, such as chymotrypsin and granzyme B. We tested a few compounds with the serine protease α-chymotrypsin. The inhibitors tested were PhPr-Val-Ala-AAsp-EP-CH_2CH_2Ph (**23a**), PhPr-Val-Ala-AAsp-*trans*-EP-$COOCH_2Ph$ (**23d**), Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**, both isomers), and Cbz-Leu-Glu-Thr-AAsp-EP-COOEt (**26c**, both isomers). No inhibition of chymotrypsin was observed after 90 minutes of incubation with an inhibitor concentration of 223 μM. Granzyme B is thus far the only major mammalian serine protease which cleaves substrates that contain a P1 Asp. Since it can also activate caspases and trigger apoptosis, it was essential to test our inhibitors with this enzyme. Five inhibitors were tested with granzyme B by Z. Wang and Dr. Froelich at Northwestern. The inhibitor Cbz-Leu-Glu-Thr-AAsp-(*S,S*)-EP-COOEt (**26c**, 500 μM) was totally inactive after incubation with enzyme for 1 hour. With PhPr-Val-Ala-AAsp-(*S,S*)-EP-$COOCH_2Ph$ (**23d**), some inhibition was seen over a period of four hours (kobs/[I] < 0.3 $M^{-1}s^{-1}$). Even less inhibition was observed with Cbz-Asp-Glu-Val-AAsp-EP-COOEt (**25c**, kobs/[I] < 0.1 $M^{-1}s^{-1}$). The aza-peptide epoxide Cbz-Ile-Glu-Thr-AAsp-(*S,S*)-EP-COOEt (**27c**) did show some inhibition, but was a very weak competitive inhibitor (IC_{50} = 105 μM). The activity of the enzyme dropped quickly in the presence of the inhibitor and remained almost constant with time. Time-dependent inhibition (if any) was not easily observed ($k_{obs}/[I]$ = 0.65 $M^{-1}s^{-1}$). Coincidently, the best and preferred substrate sequence for granzyme B is Ile-Glu-Pro-Asp, with granzyme B almost exclusively preferring isoleucine at P4.(Nicholson and Thornberry 1997)

Dr. B. Dunn, B. Beyer, and J. Clemente at the U. Florida tested six of our most potent compounds with a variety of aspartyl proteases, including porcine pepsin, human cathepsin D, plasmepsin 2 from *P. falciparum*, HIV-1 protease, and the secreted aspartic proteinase 2 (SAP-2) from *Candida albicans*. After incubation of aza-peptide epoxides with enzyme for 20 minutes (30 minutes incubation for HIV-1), no inhibition of more than 20 percent was observed.

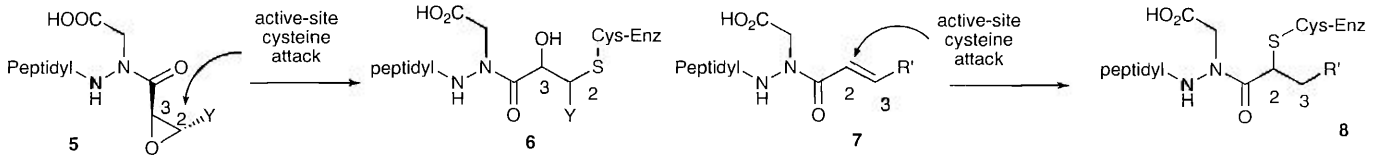
In summary, *aza-peptide epoxide inhibitors are highly specific for clan CD cysteine proteases.* Effectively, they are non-inhibitors of serine proteases, clan CA cysteine proteases, threonine, and aspartate proteases. In contrast, other widely used caspase inhibitors are not specific at all. For example, Cbz-DEVD-CMK reacts potently with caspase 3 (k_2/K_I = 1,000,000 $M^{-1}s^{-1}$), but also is quite reactive with cathepsin V (355,000 $M^{-1}s^{-1}$), cathepsin L (36,000 $M^{-1}s^{-1}$), cathepsin B (5200 $M^{-1}s^{-1}$), cathepsin S (28,100 $M^{-1}s^{-1}$), papain (1750 $M^{-1}s^{-1}$), and legumain (660 $M^{-1}s^{-1}$) (Rozman-Pungercar, Kopitar-Jerala et al. 2003). Aza-peptide epoxides are more selective than halomethyl ketones as recent experiments have demonstrated that biotinylated Cbz-VAD-FMK and Ac-YVAD-CMK can label cathepsin B and cathepsin H (Schotte, Declercq et al. 1999; *Gray, Haran et al. 2001).*

Stability. Aza-peptide epoxides exhibit considerable stability in buffer solutions. The caspase inhibitor Cbz-Leu-Glu-Thr-AAsp-(*S,S*)-EP-$COOCH_2Ph$ (**26d**, 488 nM) was incubated in buffer containing DTT (50 mM Hepes, 0.1% (w/v) CHAPS, 10 mM DTT, at pH 7.4) for 32 minutes, and then substrate (Ac-DEVD-AFC, 100 μM) and enzyme (caspase 6, 10 nM) were added and the reaction was monitored. The inhibitor was equally as potent as when not incubated with DTT. Aza-peptide epoxides designed for dipeptidyl peptidase I (DPPI) have a half-life ($t_{1/2}$) of 350 min at pH 7 and 7.5. Aza-peptide epoxides designed for legumain show no decomposition after storage for 24 hrs in buffer solutions. One advantage of epoxysuccinyl peptide inhibitors, reported in the literature, is their stability under physiological conditions toward simple thiols (Meara and Rich 1996). At physiological conditions, E-64 does not react with 100 mM cysteine nor does it inactivate lactate dehydrogenase, a non-proteolytic thiol-dependent enzyme (Barrett, Kembhavi et al. 1982).

Binding Mode and Mechanism. X-ray crystal structures of caspase 1 inhibited by two aza-peptide epoxides PhPr-Val-Ala-AAsp-EP-$COOCH_2Ph$ and PhPr-Val-Ala-AAsp-EP-CH_2CH_2Ph have also been determined (Ron Rubin, Parke-Davis now Pfizer, unpublished results). Nucleophilic attack by the active site Cys 285 of caspase 1 occurs at the C-2 position of the epoxide (**5**) resulting in a thioester linkage between the enzyme and the inhibitor (**6**). It is clear that the active site cysteine can attack functional groups quite distant from the side chain of the P1 aza-amino acid residue. We had expected the site of nucleophilic attack to be the C-3 position of the epoxide ring. Usually in transition-state inhibitors, such as peptide aldehydes, the active site cysteine adds to the aldehyde carbonyl group that is equivalent to the scissile peptide carbonyl group in a substrate.

The selectivity of aza-peptide epoxides for clan CD cysteine proteases is a novel feature of these inhibitors. We propose that our aza-peptide epoxides cannot bind in a suitable orientation for effective irreversible inhibition in the active sites of papain and other clan CA cysteine proteases, due to the inability of the fairly rigid aza-peptide epoxysuccinate moiety to bind properly near the enzyme's catalytic residues. The enzymes in clan CD and clan CA have varying active site topologies, such that the caspase substrate-binding

region is quite open and shallow with several binding pockets, while papain has a canyon-like binding site. In most caspase inhibitor complexes, the peptide chain of the inhibitor makes antiparallel beta-sheet interactions with several residues in the active site of the caspases. The more open active site in the caspases coupled with the strict specificity for a P1 Asp residue allows the aza-Asp side chain of the aza-peptide epoxide to bind in the S1 pocket. This positions the epoxide moiety in close proximity to His 237 and the nucleophilic Cys 285 where covalent bond formation can occur. However, in papain, specificity is determined by interaction with the S2 subsite and a loop structure on the wall of the canyon, which contains the active site histidine, restricts possible binding modes of inhibitors. Papain family enzymes form parallel β-sheet interactions between the enzyme and the inhibitor and use an Asn residue to orient the histidine properly in the active site. The caspases only have a catalytic dyad, but it is hypothesized that some caspases do use the backbone of residue 177 to create a catalytic triad (Stennicke and Salvesen 1998). The distance between the catalytic histidine and cysteine in caspases is longer which permits rotation of the histidine, and caspases only use one hydrogen bond to stabilize the oxyanion hole versus two hydrogen bonds in papain. These active site differences may contribute to the difference in specificity and reactivity observed for aza-peptide epoxides with caspases and clan CA enzymes. In the future, we hope to obtain additional crystal structures to confirm our mechanistic hypothesis with other caspases. We were quite surprised at the high specificity of these inhibitors for clan CD cysteine proteases and expected at least some reactivity toward other cysteine proteases, which was not observed.



Aza-peptide Michael Acceptors. We next decided to extend the aza-peptide epoxide design (**5**) to aza-peptide Michael acceptor inhibitors (**7**). We synthesized over forty derivatives, a few of which are shown in Table 6. Second-order inhibition rates (k_2 values in $M^{-1}s^{-1}$) are shown for caspase 3, 6, and 8. We have also synthesized inhibitors for legumain and gingipain which will be discussed later. Most of this data is unpublished, although we have published a brief communication (Ekici, Gotz et al. 2004). Aza-peptide Michael acceptors are potent and selective inhibitors for all the clan CD cysteine proteases examined. The aza-peptide Michael acceptors with a P1 aza-Asp residue inhibit caspases 3, 6, and 8 with k_2 values in the order of 10^6 $M^{-1}s^{-1}$ (Table 6). The DEVD and LETD sequences are optimal sequences for caspase 3 and caspase 8, respectively. The DEVD inhibitors are potent inhibitors of caspase 3 (k_2 values range up to 3,000,000), while the LETD derivatives are potent inhibitors of caspases 6 and 8 with k_2 values up to 47,600 $M^{-1}s^{-1}$ and 237,000 $M^{-1}s^{-1}$, respectively. The dibenzyl amide **9** was one of the most selective compound among the caspases 3, 6, and 8, where it inhibits caspase 3 588-fold more potently than caspase 6 and 349-fold more potently than caspase 8. The tetrahydroquinoline derivative **10** was a selective caspase 8 inhibitor with a 16-fold selectivity over caspase 3 or 6. The IET inhibitor **11** was fairly selective for caspase 6.

Table 6.

		caspase 3	caspase 6	caspase 8
Cbz-Asp-Glu-Val-AAsp-CH=CH-COOEt	*trans*	2,130,000 $M^{-1}s^{-1}$	35,575	272,960
Cbz-Asp-Glu-Val-AAsp-CH=CH-COOEt	*cis*	1,060,000	11,000	181,000
Cbz-Asp-Glu-Val-AAsp-CH=CH-$COOCH_2Ph$	*trans*	1,700,000	8,470	121,000
Cbz-Asp-Glu-Val-AAsp-CH=CH-$CONHCH_2Ph$	*trans*	1,750,000	3,210	78,235
Cbz-Asp-Glu-Val-AAsp-CH=CH-$CONHCH_2$-4-F-Ph	*trans*	2,100,000	4,400	85,100
Cbz-Asp-Glu-Val-AAsp-CH=CH-$CONHCH_2CH_2Ph$	*trans*	1,950,000	3,470	129,000
Cbz-Asp-Glu-Val-AAsp-CH=CH-$CON(CH_3)CH_2Ph$	*trans*	2,640,000	9,500	90,300
Cbz-Asp-Glu-Val-AAsp-CH=CH-$CON(CH_3)CH_2CH_2Ph$	*trans*	1,180,000	4,000	31,900
Cbz-Asp-Glu-Val-AAsp-CH=CH-$CON(CH_2Ph)_2$	*trans* (**9**)	**3,000,000**	5,100	8,600
Cbz-Asp-Glu-Val-AAsp-CH=CH-CON-tetrahydroquinoline	*trans*	2,300,000	5,700	118,000
Cbz-Leu-Glu-Thr-AAsp-CH=CH-COOEt	*trans*	5,560	18,700	237,000
Cbz-Leu-Glu-Thr-AAsp-CH=CH-$COOCH_2Ph$	*trans*	4,600	47,600	98,400
Cbz-Leu-Glu-Thr-AAsp-CH=CH-$CONHCH_2$-4-F-Ph	*trans*	4,490	3,100	171,000
Cbz-Leu-Glu-Thr-AAsp-CH=CH-CONHPh	*trans*	4,700	11,400	176,000
Cbz-Leu-Glu-Thr-AAsp-CH=CH-$CON(CH_2Ph)_2$	*trans*	8,630	14,100	129,000
Cbz-Leu-Glu-Thr-AAsp-CH=CH-CON-tetrahydroquinoline	*trans* (**10**)	12,100	13,000	**216,000**
Cbz-Leu-Glu-Thr-AAsp-CH=CH-$CON(CH_3)CH_2$-1-Napth	*trans*	11,200	21,700	179,000
Cbz-Ile-Glu-Thr-AAsp-CH=CH-$CON(CH_2Ph)_2$	*trans* (**11**)	9600	**83,900**	39,500

Our proposed mechanism of inhibition involves attack at carbon 2 of the Michael acceptor 7 to give the thioether product **8**. It should be noted that the numbering of epoxides and Michael acceptors differ (see **5** and 7). We favor C-2 attack, since the dienyl derivative Cbz-Val-AAsp-CH=CH-CH=CH-CH_3 designed for caspases showed no inhibitory potency toward caspases and was also stable when tested for reactivity with DTT. In addition, it has previously been shown that simple acrylates are more reactive than simple acrylamides to thiol nucleophiles such as glutathione (Freidig, Verhaar et al. 1999). Attack at C-2 is Michael addition to an acrylate where R' = an ester, while attack at C-3 is addition to an acrylamide.

We further investigated the inhibition mechanism by conducting an NMR study of the reaction of the inhibitors with the thiol nucleophile benzyl mercaptan. The inhibitor Cbz-Asp-Glu-Val-AAsp-CH=CH-COOEt was reacted with benzyl mercaptan, and the reaction was monitored using ^1H NMR over 48 hours. The formation of a new signal at 4.05 ppm was observed with the simultaneous disappearance of the vinyl proton signal at 6.63 ppm. The observed spectrum was consistent with a thioether adduct that is formed by the attack of the benzyl mercaptan at the C-2 carbon (7). An attack at the C-3 carbon would result in a chemical shift of the C-3 hydrogen at 3.59 ppm as predicted by the additivity rules (Buergin-Schaller, Arnold et al. 1995) used in ChemDraw. There was no further change in the spectrum after 48 hours, which indicates that probably a stable thioether adduct had formed. The evidence for the mechanism is compelling, but not infallible, and we would like to obtain a crystal structure of one of these adducts.

Stability. The aza-peptide Michael acceptors were tested for their reactivity toward thiol nucleophiles such as the thiol DTT contained in the assay buffer conditions. The stability of a representative group of compounds was studied by monitoring the UV spectrum of buffer solutions of the inhibitors at 250 nm, at 25 °C. The buffer solutions contained 10 mM DTT and the pH range was 5.8-7.2. The half-lives ($t_{1/2}$) for the esters (7, R = COOR) range from 3-10 min, whereas the monosubstituted amides (7, **R** = CONHR) were less reactive with half-lives of 20-58 min. A caspase specific disubstituted amide analog (R = $CONR_2$) had a half-life of 116 min, whereas a similar disubstituted amide analog designed for legumain was stable when tested over a period of 15 hours. Additional kinetic studies are proposed to elucidate the structural features which result in additional stability in aza-peptide Michael acceptor inhibitors.

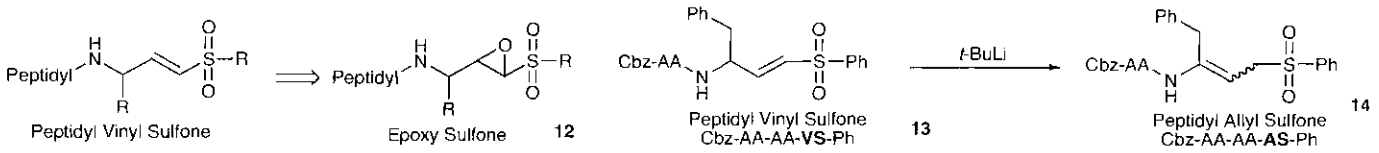
Legumain Aza-peptide Inhibitors. We have applied both the aza-peptide epoxide and Michael acceptor design to legumain and synthesized about 30 aza-peptide inhibitors. Table 7 lists kinetic data with both *S. mansoni* and pig kidney legumain. Most of the compounds are epoxides, but the last two inhibitors are Michael acceptors. The work with the epoxides has been published (James, Gotz et al. 2003) and one legumain inhibitor has been used to develop a cytochemical assay for legumain activity in pig kidney and mouse skin (Zeeuwen, van Vlijmen-Willems et al. 2004). With the parasitic blood fluke *Schistosoma mansoni* legumain, Connor Caffrey and Jim McKerrow at UCSF measured IC_{50} values, and $k_{obs}/[I]$ values for some of the more potent inhibitors. Only limited data with the pig kidney enzyme is available since our collaborator, Dr. Alan Barrett of the Strangeways Laboratory (Cambridge, England), shut down his wet lab during the course of the project to devote full time to the protease web site MEROPS. (http://merops.sanger.ac.uk/).

Table 7.	*S. mansoni* (IC_{50} & $k_{obs}/[I]$)		pig kidney ($k_{obs}/[I]$)
Cbz-Ala-Ala-AAsn-(*S,S*)-EP-COOEt	53 nM	17,400 $M^{-1}s^{-1}$	43,000 $M^{-1}s^{-1}$
Cbz-Ala-Ala-AAsn-(*R,R*)-EP-COOEt	788		25,200
Cbz-Ala-Ala-AAsn-(*cis*)-EP-COOEt			140
Cbz-Ala-Ala-AAsn-(*S,S*)-EP-$COOCH_2Ph$	47	15,300	26,700
Cbz-Ala-Ala-AAsn-(*S,S*)-EP-$COOCH_2CH_2Ph$	45		
Cbz-Ala-Ala-AAsn-(*S,S*)-EP-$CON(nBu)_2$	68		
Cbz-Ala-Ala-AAsn-(*S,S*)-EP-$CON(CH_3)CH_2Ph$	63		
Cbz-Ala-Ala-AAsn-(*trans*)-EP-CH_2CH_2Ph	70		
Cbz-Ala-Ala-AAsn-(*trans*)-EP-Ph-4-Cl	90		
Cbz-Ala-Ala-AAsn-trans-CH=CH-$CON(CH_3)CH_2$-1-Napth	35	16,930	
Cbz-Ala-Ala-AAsn-trans-CH=CH-$CON(Bzl)_2$	45	5,800	

We tested both classes of legumain inhibitors with other clan CD cysteine proteases, such as caspases, gingipain, and clostripain, and clan CA proteases including papain, calpain, and cathepsin B and found little to no cross-reactivity.

Allyl Sulfones-A New Class of Inhibitors For Clan CA Cysteine Proteases. Peptidyl vinyl sulfones are well known irreversible inhibitors for clan CA cysteine proteases and the proteasome (Palmer, Rasnik et al. 1995; Powers, Asgian et al. 2002). We attempted to combine the vinyl sulfone design with the epoxide inhibitors described early and tried to synthesize peptide epoxy sulfones (**12**). When we took a simple dipeptide

vinyl sulfone (**13**) and treated it with *t*-BuLi in the presence of an oxidizing agent, we obtained a new compound. This new compound was subsequently shown to be a peptidyl allyl sulfone (**14**), which resulted by isomerization of the vinyl sulfone double bond.



The new peptidyl allyl sulfones were tested first with a variety of clan CA cysteine proteases (calpain I, papain, cathepsin B, L, cruzain, and rhodesain), since vinyl sulfones are fairly reactive with the group of cysteine proteases. Inhibition date is shown in Table 8. This new class of compounds shows moderate rates of calpain and papain inactivation and good rhodesain and cruzain inactivation. The oxazoline, also a potential product from the base catalyzed isomerization reaction, was also a slow inhibitor of calpain I. Interestingly, the allyl sulfones were more reactive than the vinyl sulfone containing the same sequence. At present, we don't know if these inhibitors are specific for clan CA cysteine protease or whether they can be extended to clan CD enzymes. Currently, we are not developing these inhibitors further because we are focusing on aza-peptides.

			$k_{obs}/[I]$ (M^{-1} s^{-1})		IC_{50} (μM)	
Table 8.	Calpain I	Papain	Cathepsin B	Cathepsin L	Cruzain	Rhodesain
Cbz-Ala-Phe-AS-Ph	3	9	N.I.	183	>10	>10
Cbz-Val-Phe-AS-Ph	3	6	N.I.	310	6	5
Cbz-Leu-Phe-AS-Ph (A)	23	49	N.I.	700	0.06	0.04
Cbz-Leu-Phe-AS-Ph (B)	564	15	N.I.	1060	0.3	0.18
Cbz-Leu-Phe-VS-Ph	550	10	N.I.	219	2	0.5
oxazoline	13	N.I.	N.I.	N.I.	>10	>10

Allyl sulfone A is derived from the L-isomer of Phe. Allyl sulfone B is derived from the D-isomer of Phe.

Calpains are a class of calcium activated cysteine proteases. Enhanced calpain activity however has been related to cell injury due to ischemic stroke (Bartus, Elliott et al. 1995), physical damage (Seubert, Ivy et al. 1988), hypoxia (Lee, Frank et al. 1991), and peripheral neuropathy (Wang, Davis et al. 2004). Cruzain and rhodesain are clan CA cysteine proteases, which are essential for the development and survival of the parasite *Trypansoma brucei*, the protozoan parasite species that causes sleeping sickness in humans and cattle. Cathepsin B and L are lysosomal cysteine proteases and cathepsin B is associated with invasive breast tumors.

Apoptosis Assays (Specific Aim 5). Fas induced apoptosis assays in the Jurkat (MTT assay) with several aza-peptide epoxides were done in Dr. Guy Salvesen's lab by Dr. Jowita Mikolajczyk and the results are shown in Table 9. The assays were done with 0 μM (control), 10 μM, and 50 μM of inhibitor. Three fluoromethyl ketone (FMK) "caspase specific" inhibitors where also tested in this tissue culture paradigms of cell fate decisions to define which are due to caspases and which to other proteases. "The aza-peptide epoxides efficiently blocked apoptosis with the same potency as FMK inhibitors, but showed a distinct reduction in (artefactual) side effects" (from Jowita Mikolajczyk's abstract to the cell death conference this summer). The caspase 3 inhibitor (**15**) has also been shown to completely stop apoptosis in the laboratory of Dr. Jonathan Glass at Emory University using the Sytox assay (Wang, Davis et al. 2004).

Table 9. Inhibition of Fas induced apoptosis (% survival). Inh. Conc:	50 μM	10 μM	0 μM
Control			19
Cbz-Ile-Glu-Thr-AAsp-(*S,S*)-EP-CO_2Et	87	52	
Cbz-Ile-Glu-Thr-AAsp-(*S,S*)-EP-CO_2Et	88	54	
Cbz-Glu-Val-AAsp-(*S,S*)-EP-CO_2Et	83	60	
Cbz-Asp-Glu-Val-AAsp-(*R,R*)-EP-CO_2Et	24	21	
Cbz-Asp-Glu-Val-AAsp-(*S,S*)-EP-CO_2Et (**15**)	78	51	
Cbz-Val-Asp-FMK	111	113	
Cbz-Ile-Glu-Thr-Asp-FMK	98	70	
Cbz-Leu-Glu-Thr-Asp-FMK	98	64	

X-ray Structures of Caspase-Inhibitor Complexes. Five crystal structures of caspase–3: aza–peptide epoxide inhibitor complexes were reported and reveal the structural basis for the mechanism of inhibition and the specificities at the S1' and the S4 subsites. Unlike the clan CA cysteine proteases, the catalytic histidine in caspase–3 plays a critical role during protonation and subsequent ring opening of the epoxide moiety and

facilitates nucleophilic attack by the active site cysteine. The nucleophilic attack takes place on the C–3 carbon atom of the epoxide and results in an irreversible alkylation of the active site cysteine residue. A favorable network of hydrogen bonds involving the oxyanion hole, catalytic histidine and the atoms in the prime site of the inhibitor enhance the binding affinity and specificity of the aza–peptide epoxide inhibitors towards caspase–3. The studies also reveal that subtle movements of the N–terminal loop of the β–subunit occur, when the P4 Asp is replaced by a P4 Ile, whereas the N–terminal loop and the "safety catch" Asp179 are completely disordered when the P4 Asp is replaced by P4 Cbz group.

Main Protease from the the SARS coronavirus. The main protease (M^{pro}) from the coronavirus (CoV) causing severe acute respiratory syndrome (SARS) is one of the most attractive molecular targets for the development of anti-SARS agents. We reported the crystal structures of SARS-CoV M^{pro} unbound in the crystal form C2, and bound by an aza-peptide epoxide (APE) in the crystal forms C2 and $P2_12_12_1$, respectively. These structures show that the aza-peptide epoxide irreversibly inhibits SARS-CoV M^{pro} by the formation of a covalent bond between the catalytic Cys-145 S atom of the protease and the epoxide C_3 atom of the inhibitor. The aza-peptide component of APE binds in the substrate-binding regions of SARS-CoV M^{pro} in a substrate-like mode. Clues for the optimization of APE:SARS-CoV M^{pro} binding were described. In addition, the 'N-fingers' (N-terminal residues 1 to 7) of both protomers of SARS-CoV M^{pro} are well defined and the substrate-binding regions of both protomers are in the catalytically competent conformation at the crystallization pH (pH 6.5).

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